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13. ABSTRACT (Maximum 200 Words)

Hereditary predisposition to breast cancer is associate with mutations in the BRCA1 gene. BRCA1 functions as a tumor suppressor that is activated in response to DNA damage and the loss of normal BRCA1 activity leads to an increase in chromosome instability. BRCA1 is thought to respond to DNA damage incurred during S phase, but it is not clear precisely how BRCA1 recognizes DNA damage or transmits effector signals to delay cell cycle progression and allow efficient repair of damaged DNA. In this proposal, we present preliminary data that BRCA1 functions in a DNA checkpoint response for the origin of Epstein-Barr Virus DNA replication (Ori P). OriP replicates once and only once per cell cycle in synchrony with the cellular genome, and is therefore considered a valid model system for cellular replication origins. Importantly, chromosomal replication proteins, including the origin recognition complex (ORC) and the licensing helicase complex (MCMs) associate with OriP. We have recently found that BRCA1 can be recruited to OriP in response to DNA damaging agents. We propose to study the mechanism of BRCA1 recruitment to OriP, its dependence on DNA-damage induced post-translational modifications, and to investigate its function at OriP in DNA replication and plasmid maintenance. We propose that these studies will provide valuable information concerning the function of OriP at replication origins and in the control of DNA replication initiation and genome stability.

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Paul Lieberman, Ph.D., Principal Investigator

Introduction

The BRCA1 tumor suppressor protein is thought to function in multiple processes that regulate genome stability and gene regulation (8, 10, 11). In this proposal we are investigating the function of BRCA1 at a DNA replication origin. BRCA1 associates with the Epstein-Barr virus origin of DNA replication, OriP. OriP is unique metazoan replication origin with well-defined genetic parameters (6, 9). One viral encoded protein, referred to as EBNA1, binds to multiple repeats in OriP and recruits cellular replication and licensing machinery (2, 5). OriP replicates once and only once in synchrony with the S phase of the host cell (1, 12). OriP requires the function of the ORC complex and associates with the MCM complex in a cell cycle dependent manner (2, 5, 7). In this regard, OriP is regulated indistinguishably from cellular origins of replication and may serve as a valuable genetic and biochemical model for the function of cellular proteins regulating replication origin function and stability. OriP consists of two functional genetic elements referred to as the family of repeats (FR) and the dyad symmetry (DS). Replication initiates from the DS, and we have found that telomere repeat binding factors (TRFs) bind to DS, as well(3, 4). TRF2 and hRap1 were found to promote DS replication (3). TRF2 is known to interact with the MRN complex that consists of the MREll, NBS1 and RAD50 proteins (13). We now show that the MRN complex associates with DS in a cell cycle dependent manner. We also show that BRCA1 associates with DS in a cell cycle dependent manner. We hypothesize that BRCA1 and associated proteins function at OriP to maintain replication origin structure and integrity without evoking a cellular DNA damage response. In this respect, OriP is a privileged DNA site that can adopt aberrant structure without invoking DNA damage repair. This has potential significance for the role of BRCA1 and associated proteins in numerous other processes in the nucleic acid metabolism.

Original Overview. BRCA1 can be recruited to the EBV origin of plasmid replication when cells are treated with HU. We hypothesize that a BRCA1-dependent protein-modifications occurs at OriP to postpone the initiation of DNA replication. At least two post-translational modifications have been implicated in our studies, these include monoubiquitination and poly-ADP ribosylation. The objective of this study will be to determine what function BRCA1 may play in the regulation of DNA replication initiation, and what signals are required for the recruitment of BRCA1 to the replication origin.

Original Specific Aims. (1) Using BRCA1 negative cell lines and dominant negative expression plasmids we will determine if BRCA1 is important in the DNA replication or plasmid maintenance function of OriP. (2) We will determine whether BRCA1 recruitment to OriP is dependent on telomeric repeat sequences found in OriP or upon the poly-ADP ribose activity of Tankyrase or PARP1 found to be associated with the telomeric repeats in OriP. (3) We will determine whether BRCA1 leads to the post-translational modification of OriP binding proteins, and whether this leads to the dissociation of these proteins from OriP.

BODY

Key Research Accomplishments in this Funding Period

BRCA1 and BACH1 can be identified in DNA affinity purification using EBV OriP DS region. We have used DNA affinity purification with the DS region of OriP to identify cellular proteins important for replication function (Fig.1). We have used MS/MS and Western blotting analysis to identify BRCA1, BACH1, and MRE11 as components that associate with DS. The DS consists of three telomere repeats and four EBNA1 binding sites (Fig. 1A). The association of BRCA1, BACH1, and MRE11 were dependent upon the telomere repeats, suggesting that TRF2-DNA binding and protein interactions are important for recruiting these additional factors (data not shown). We also found that the BRCA1, BACH1, and MRE11 components are enriched at DS in extracts derived from cells treated with hydroxyrea (HU) (Fig. 1B). This suggests that DNA damage or cell cycle arrest stimulates complex formation at DS.

BRCA1 binds OriP in vivo by chromatin immunoprecipitation assay. We used chromatin immunoprecipitation (ChIP) assays to determine if BRCA1 is enriched at Orip in vivo (Fig. 2A). We found that BRCA1 was enriched at DS relative to other regions of the EBV genome, including the inactive OriLyt region. Most importantly, we found that BRCA1 was highly enriched at DS in cells arrested in G1/S with mimosine. These data indicate that BRCA1 associates with DS in a cell cycle dependent manner.

The MRN complex associate with OriP in a cell cycle dependent manner. We used ChIP{ assay to determine if MRE11 or NBS1 proteins associate with the DS region of OriP in a cell cycle dependent manner (Fig. 2B). We found that both MRE11 and NBS1 had a similar binding profile to BRCA1 at DS. This suggests that the MRN complex and BRCA1 colocalize to OriP DS DNA in the G1/S phase of the cell cycle.

DNA PK associates with OriP by ChIP assay, but does not have a cell cycle dependence similar to MRN or to BRCA1. DNA PK has been implicated in numerous DNA metabolic processes and can be localize to cellular telomeres and sites of DNA damage. We used the ChIP assay to measure DNA PK interactions with DS in vivo. We found that DNA PK can be recruited to the DS region of OriP. However, in contrast to BRCA1 and MRN complex, DNA PK binding to DS was not enriched in G1/S phase of the cell cycle. Therefore, DNA PK associates with DS, but does so independently of BRCA1 and MRN.

SiRNA depletion of BRCA1 or BACH1 leads to a potent inhibition of OriP DNA replication activity. The contribution of BRCA1 and BACH1 to OriP replication was assayed using a transient DNA replication assay. Plasmid DNA containing OriP replication sequences and expressing EBNA1 protein was cotransfected with siRNA against BRCA1, BACH1, or control luciferase gene (Fig. 3). DNA replication was assayed 72 hrs post-transfection using the Dpn1 resistance assay. Briefly, plasmid DNA was recovered by Hirt extraction and then digested with BamHI to linearize total DNA or with BamHI and DpnI. DpnI will digest DNA replicated in E. coli, but will not digest DNA replication in mammalian cells due to difference in adenosine methylation patterns. The siRNA directed against BRCA1 and BACH1 were shown to deplete

their target proteins by greater than 80%. Depletion of BRCA1 and BACH1 led to a significant inhibition of OriP replication (5 fold for BRCA1). These findings suggest that BRCA1 contributes to the OriP replication function.

Distinct histone modifications at OriP. Chromatin structure and histone modifications at OriP have been examined using indirect end-labeling and ChIP assays. We have found that nucleosomes are positioned adjacent to DS and that they are constitutively enriched for histone H3 K4 methylation (H3 mK4) relative to most other regions of the EBV genome (Fig. 4). Histone H3 mK4 methylation has been shown to be induced by mono-ubiquitination of histone H2B through a RAD6-dependent pathway. The mechanism of histone methylation at OriP remains unclear, and its relation to BRCA1 recruitment is under investigation.

Reportable Outcomes

Manuscripts: One submitted in collaboration with Dr. R. Shiekhattar (The Wistar Institute) describing the role of BRCA1 and BRCA2 in OriP DNA replication using siRNA directed against BRCA1 and BRCA2.

Conclusions

We conclude that BRCA1 and MRE11/NBS1 associate with the DS region of OriP during the G1/S border of the cell cycle. This association is dependent on the telomere repeat binding factor sites in DS, and therefore is probably a result of cell cycle interactions between TRF2/hRap1 with BRCA1 and MRN complex. The BRCA1 protein is required for OriP replication based on siRNA depletion studies. These results indicate that BRCA1 and MRN associate with the EBV replication origin during the normal cell cycle, and not just during DNA damage or S phase arrest. This is consistent with the known role of MRN complex at stalled replication forks. These results also imply that BRCA1 and MRN colocalize with TRF2 at telomeres, although this has not been demonstrated. However, it remains unclear what the function of MRN and BRCA1 is at the replication origin of EBV or at telomeres. Several hypotheses will be tested in the immediate future. The Mre11 protein functions as an endo and exonuclease that can resolve the invading single stranded DNA in the D-loop of holliday junctions and telomeric T-loops. We will test the hypothesis that homologous recombination is an important initiating event in OriP replication.

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APPENDICES

Figures 1-4

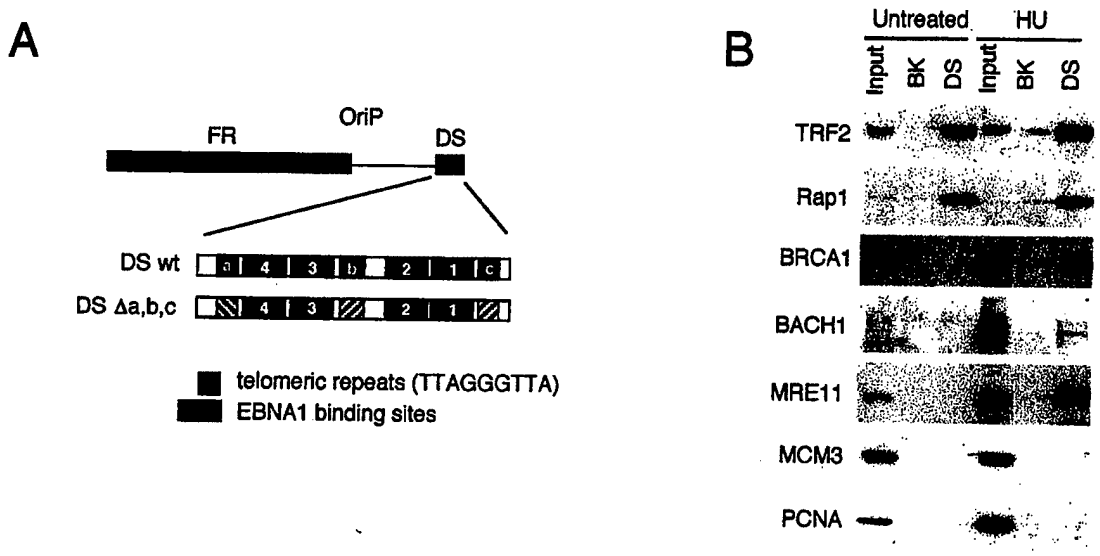
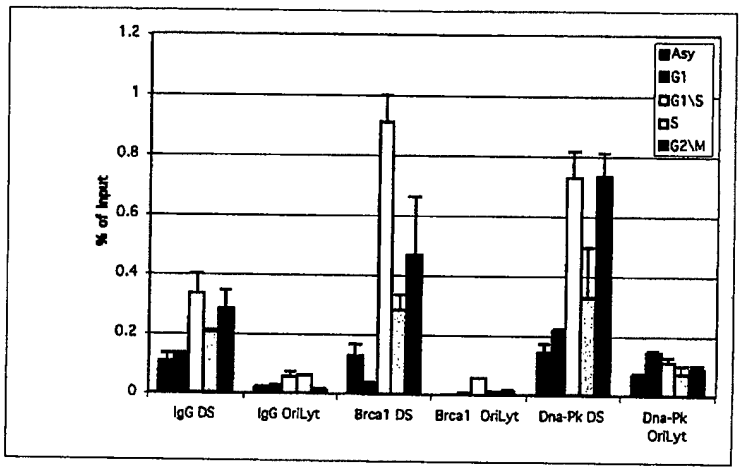


Figure 1. BRCA1 associates with DS in response to HU induced cell cycle arrest. A) Schematic of EBV OriP genetic elements and the telomere repeat mutants (DSΔa,b,c) used in some of these studies. B) DNA affinity purification with DS DNA or control BKS DNA was used to isolate proteins from Raji cell extracts untreated or treated with 50 μM hydroxyurea (HU). Affinity purified proteins were analyzed by Western blot with antibodies to TRF2, hRap1, BRCA1, BACH1, MRE11, MCM3, and PCNA, as indicated.



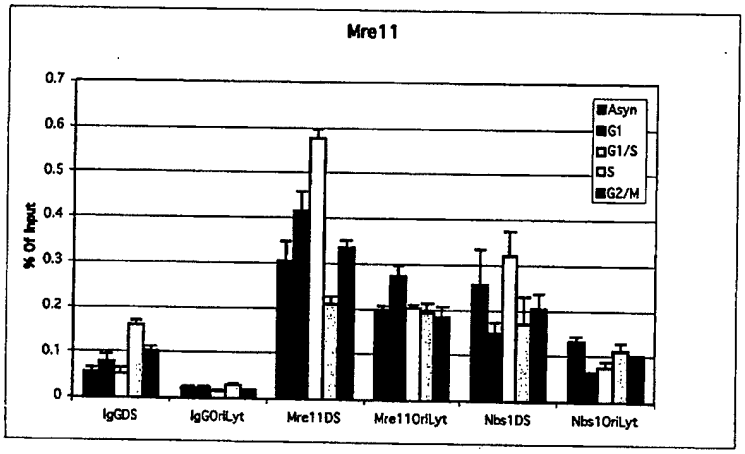


Figure 2. BRCA1, MRE11, NBS1, and DNA-PK associate with DS in a cell cycle-depdendent manner. Antibodies to BRCA1, MRE11, NBS1, or DNAPK were used in chromatin immunoprecipitation assays (ChIP) and measured by Real-Time PCR. Raji cells were synchronized in G1 by Nocodozole release, G1/S by mimosine block, S by aphidicolin block, and G2/M with nocodozole block.

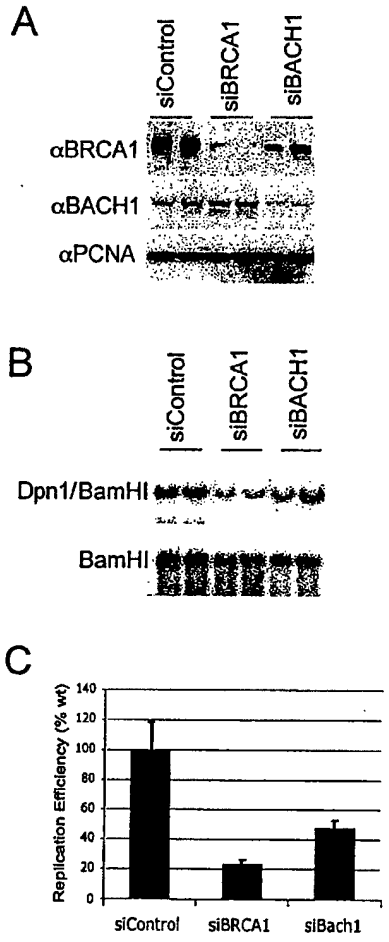


Figure 3. DNA replication activity of OriP after siRNA deplection of BRCA1 or BACH1. A) HeLa cells were cotransfected with pOriP and siRNA directed against control luciferase, BRCA1, or BACH1 as indicated and assayed by Western blot for protein levels of BRCA1, BACH1, or control PCNA. B) Replication assays of pOriP in HeLa cells transfected with siRNA as above. OriP plasmid was extracted by Hirt lysis and then digested with DpnI/BamHI or BamHI as indicated. The DpnI/BamHI resistant DNA represents replicated plasmid DNA. C) Quantification of replication measured in panel B as the ratio of DpnI/BamHI to BamHI DNA.

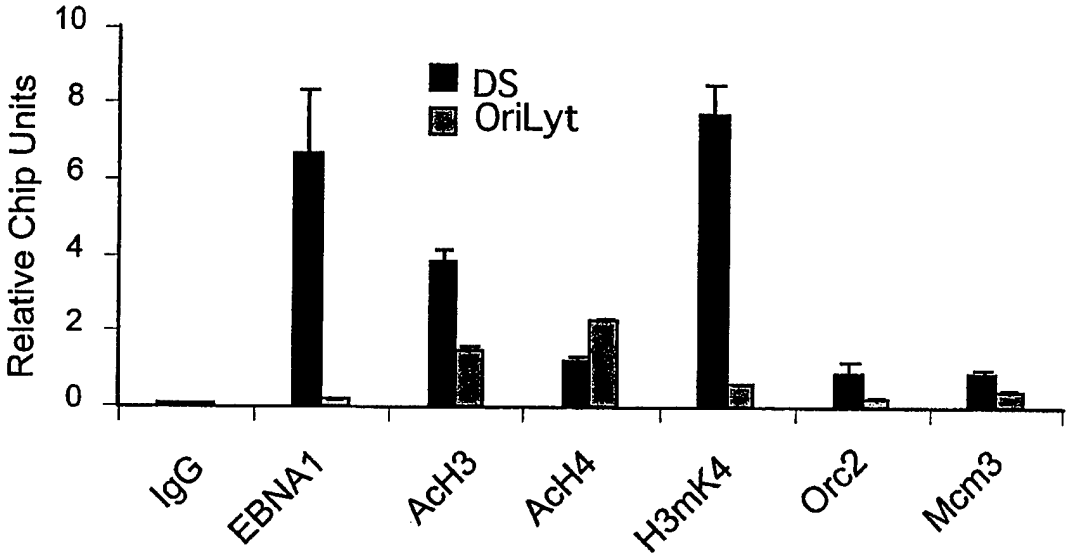


Figure 4. Histone H3 K4 methylation is enriched at DS. Real time PCR analysis of ChIP DNA from EBV positive Raji cells. DNA assayed was either DS or OriLyt region of EBV genome. Antibodies for ChIP were EBNA1, AcH3, AcH4, H3mK4, ORC2, MCM3, or control IgG, as indicated.